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N-Terminal Amino Acid Sequences of 440 kDa Hemoglobins of the Deep-sea Tube Worms, *Lamellibrachia* sp.1, *Lamellibrachia* sp.2 and Slender vestimentifera gen. sp.1 Evolutionary Relationship with Annelid Hemoglobins

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ABSTRACT—The deep-sea tube worm *Lamellibrachia,* belonging to the phylum Vestimentifera, contains two types of extracellular hemoglobins, a 3,000 kDa hemoglobin and a 440 kDa hemoglobin. The latter hemoglobin is composed of four heme-containing chains with molecular masses of 16–18 kDa. We have collected *Lamellibrachia* sp.1, *Lamellibrachia* sp.2 and Slender vestimentifera gen. sp.1 from the deep-sea cold-seep or hydrothermal areas at a depth of 1100–1400 m. The four constituent chains of the 440 kDa hemoglobin were isolated from each of the three tube worms by reverse-phase chromatography, and the N-terminal amino acid sequences of 16–44 residues were determined by automated protein sequencer. The amino acid sequences of the homologous chains showed high homology (76–85%), suggesting that they are closely related. The sequences also showed 45–49% homology with annelid hemoglobins. A phylogenetic tree constructed from hemoglobin sequences showed that the tube worm *Lamellibrachia,* the polychaete *Tylorrhynchus* and the oligochaete *Lumbricus* diverged from a common ancestor at almost the same time, about 450 million years before present.

INTRODUCTION

The deep-sea tube worms *Riftia* and *Lamellibrachia,* belonging to the phylum Vestimentifera, are found in hydrothermal vent or cold seeps at a depth of 600–2,500 m. These animals are sustained by mutual symbiosis with sulfide-oxidizing bacteria [3], and their blood, containing abundant extracellular hemoglobin, has a function to transport sulfide (H_2S) to internal bacterial symbionts, as well as to facilitate oxygen transport compatible with high oxygen demand [1].

The tube worm *Lamellibrachia* sp.1, referred as *Lamellibrachia* sp. in previous papers [17–20], has two types of giant extracellular hemoglobins, a 3,000 kDa hemoglobin and a 440 kDa hemoglobin. The former hemoglobin is composed of four 16–18 kDa heme-containing chains and two 24–26 kDa linker chains, while the latter consists of only four heme-containing chains. Two of the four heme-containing chains are common to both hemoglobins. So far, we isolated most of the constituent chains of the two hemoglobins by a reverse-phase chromatography, and determined the complete amino acid sequences of a 16 kDa heme-containing chain [18] and a 24 kDa linker chain [19]. The sequence results suggested that the hemoglobin of *Lamellibrachia* sp.1 is closely related to those of annelids.

In this report, we isolated four heme-containing chains of 440 kDa hemoglobin newly from the two tube worms, *Lamellibrachia* sp.2 and Slender vestimentifera gen. sp.1, and determined the N-terminal sequences of all the chains. Phylogenetic relationship among the tube worms and annelids is

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discussed.

MATERIALS AND METHODS

The tube worm *Lamellibrachia* sp.1 and Slender vestimentifera gen. sp.1 (both undescribed) were collected from the cold-seep area located off Sagami Bay at a depth of 1110–1170 m, Japan, by a Japanese submersible *SHINKAI 2000*. *Lamellibrachia* sp.2 (undescribed) and Slender vestimentifera gen. sp.1 were collected from the hydrothermal area of the Okinawa Trough, eastern part of the Iheya Ridge at a depth of 1405 m, Japan.

Hemoglobin was purified from frozen animals as described previously [17]. Two hemoglobin components, a 3,000 kDa hemoglobin and a 440 kDa hemoglobin, were isolated on a gel filtration column of Superose 12 (Pharmacia, 1×30 cm), in 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 0.5 ml/min. The constituent polypeptide chains of the reduced 440 kDa hemoglobin were separated by a Cosmosil $5C_{18}$-300 column (4.6×150 mm, Nacalai Tesque) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid [17].

The amino acid sequence of carboxymethylated protein was determined directly by use of an automated sequencer (Applied BioSystems 477A Protein Sequencer).

RESULTS AND DISCUSSION

We separated 3,000 kDa and 440 kDa hemoglobins from the three tube worms, *Lamellibrachia* sp.1, *Lamellibrachia* sp.2 and Slender vestimentifera gen. sp.1, by a high-performance gel filtration column of Superose 12. A typical elution profile for the latter species is shown in Fig. 1. The 3,000 kDa hemoglobin is susceptible to dissociation, and its several dissociation products emerge after the 440 kDa hemoglobin as small peaks. The hemoglobins of *Lamellibrachia* sp.1 and Slender vestimentifera gen. sp.1 were isolated mainly in the oxygenated form, but that of *Lamellibrachia* sp.2 was almost in the met (oxidized) form.

The extracellular giant hemoglobins of annelids and tube worms comprise four heme-containing chains, which can be separated into two distinct

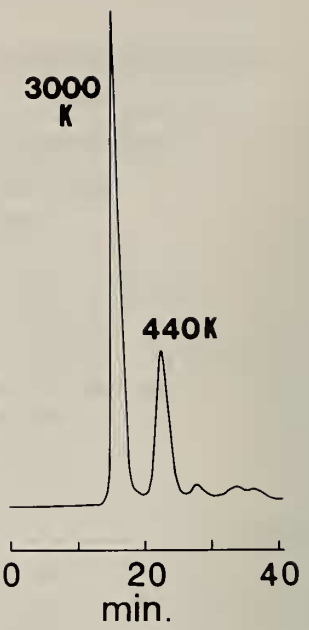

Fig. 1. Separation of the 3,000 kDa and 440 kDa hemoglobins of Slender vestimentifera gen. sp.1 on a gel filtration column of Superose 12. The column was equilibrated and eluted with 50 mM phosphate buffer containing 150 mM NaCl at a flow rate of 0.5 ml/min. Absorbance was monitored at 540 nm.

strains, A and B as suggested by Gotoh *et al.* [7]. However, the nomenclature of the chains is not unified among the researchers, so it is strongly recommended to propose a common name, like alpha and beta chains of vertebrate hemoglobins, for the corresponding chains to make clear their evolutionary relationship. Gotoh *et al.* [8, 9] were the first to propose a common name, a, A, b and B for each chain (see Table 1). But not all of the researchers agreed with the nomenclature, mainly because the difference between capital and small characters is difficult to appreciate in the spoken language [9]. Therefore we modified the nomenclature proposed by Gotoh *et al.* [8, 9], and introduced a new name (A1, A2, B1 and B2) for each of the heme-containing chains, based on the amino acid sequence homology between the chains, in this paper (Table 1).

The four heme-containing chains of the tube worm 440 kDa hemoglobin were separated by a reverse-phase chromatography. A typical elution profile for Slender vestimentifera sp.1 is shown in

TABLE 1. Nomenclature for four heme-containing chains of annelid and tube worm hemoglobins

Source	Chain Name (Earlier Nomenclature)				Reference
	Strain A		Strain B		
Lumbricus	I	II	III	IV	Vinogradov *et al.* [23]
Lumbricus	d	b	c	a	Fushitani *et al.* [5]
Tylorrhynchus	I	IIA	IIC	IIB	Suzuki *et al.* [13]
Lamellibrachia	I	III	IV	II	Suzuki *et al.* [17]
Proposed name	a	A	b	B	Gotoh *et al.* [8, 9]
	A1	A2	B1	B2	This paper

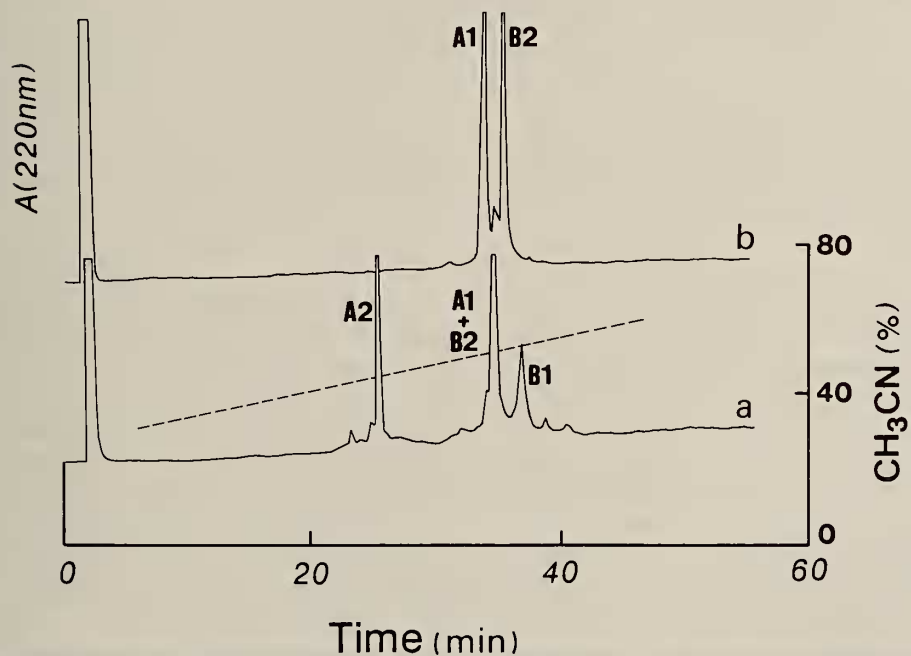

FIG. 2. Separation of the constituent chains of the 440 kDa hemoglobin from Slender vestimentifera gen. sp.1 by reverse-phase chromatography. The column (Cosmosil $5C_{18}$-300) was eluted with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. (a), reduced 440 kDa hemoglobin; (b), rechromatography of chains A1 and B2 after carboxymethylation.

Fig. 2. In this case, chains A1 and B2 were not separated (Fig. 2a), but they were separated by rechromatography after carboxymethylation of cysteine residues (Fig. 2b).

The N-terminal amino acid sequences of 16–44 residues of the isolated chains of tube worm 440 kDa hemoglobin were determined by an automated protein sequencer and aligned in Fig. 3 with those of the polychaete *Tylorrhynchus* [13, 14, 16, 21] and the oligochaete *Lumbricus* [5, 12]. The amino acid sequences (total 134 residues compared) of the homologous chains from the three tube worms showed high homology (76–85%) with each other, suggesting that they are closely related. In addition, the sequence of tube worm hemoglobin has significant homology (45–49%) with those of annelid hemoglobins, which is comparable to the homology (43%) between the

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                          1        10        20        30        40
A1 chain   Lam.sp.1         DcNILQRLKVKMQWAKAYG  FGAERAKFGNSLWTSIFNYAP
           Lam.sp.2        EKcNDLERIKVKMQWAKAYS  FSANRAKFGDALWANVFNYAP
           Ves.sp.1        DNcNILQRIKMKMQWGKAYG  TGAKRAEFGDALWANVFNYAP
           Tyl.            TDcGILQRIKVKQQWAQVYS  VGESRTDFAIDVFNNFFRTNP
           Lum.             EcLVTEGLKVKLQWASAFG  HAHQRVAFGLELWKGILREHP
A2 chain   Lam.sp.1        YEcGPLQRLKVKRQWAEAYG  SGNDREEFGHFIWTHVF
           Lam.sp.2       DHVcGPLQRLKVKRQWAEAYG  SGNRREDFGHYIWAHVF
           Ves.sp.1      DTHVcGPLQRLKVKRQWAEAYG  SGGRREDFGHYIWAHVF
           Tyl.          SSDHcGPLQRLKVKQQWAKAYG  VGHERVELGIALWKSMF
           Lum.           KKQcGVLEGLKVKSEWGRAYG  SGHDREAFSQAIWRATF
B1 chain   Lam.sp.1         SKFcSEGDATIVIKQW
           Lam.sp.2       EASDHcHYEDAEIVMKEW
           Ves.sp.1     TVVSDDcSYEDADIVMKEW
           Tyl.            DTCcSIEDRREVQALW
           Lum.         DEHEHCcSEEDHRIVQKQW
B2 chain   Lam.sp.1        SSNScTTEDRREMQLMWANVWSAQFTGRRLAIAQAVFKDLFA
           Lam.sp.2          SNHcTTEDRREMQLMWGNVWSAQFTGRRLAIAQAVFKDLFD
           Ves.sp.1          SNHcTTEDRKEMQIMWSNVWHAQFTGRRLAIAQAVFNDLFA
           Tyl.              DDCcSAADRHEVLDNWKGIWSAEFTGRRVAIGQAIFQELFA
           Lum.         ADDEDCcSYEDRREIRHIWDDVWSSSFTDRRVAIVRAVFDDLFK
                                *         *
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Fig. 3. Alignment of N-terminal amino acid sequences of four heme-containing chains of three tube worms with those of *Tylorrhynchus* and *Lumbricus*. Asterisks indicate the invariable residues in all chains. Lam. sp.1, *Lamellibrachia* sp.1; Lam. sp.2, *Lamellibrachia* sp.2; Ves. sp.1, Slender vestimentifera gen. sp.1; Tyl., *Tylorrhynchus*; Lum., *Lumbricus*.

polychaete and oligochaete hemoglobins.

In annelid-like giant hemoglobins, Cys residues play a particularly important role in the subunit assembly of the giant molecule. They are all participating in either intra- or interchain disulfide bridges [5, 15]. All of the chains of the tube worm hemoglobins conserves Cys-7 that would be used for the formation of intrachain disulfide bridge.

The deep-sea tube worms were placed in a new phylum, Vestimentifera, on the basis of their unique outward appearance, such as the very long trunk region and absence of a mouth, gut and anus [10]. Since there is no fossil record on tube worms, it is very hard to get an evolutionary relationship among the tube worms and other invertebrate animals directly. So far both of the 18S ribosomal RNA sequence [4] and hemoglobin sequence [17, 18] suggest that the tube worm and annelid are closely related. In order to get more information on the evolution of the tube worms, we constructed a phylogenetic tree for the hemoglobin sequences by an unweighted pair group clustering method (Fig. 4). Standard errors are given at each branching point, to help evaluation of the tree [11].

Fig. 4a shows a phylogenetic tree constructed from the partial hemoglobin sequences (total 134 residues) of the four chains of the three tube worms and two annelids (see Fig. 3). The tree apparently indicates that the tube worms, polychaete *Tylorrhynchus* and oligochaete *Lumbricus* diverged from a common ancestor at almost the same time, and the radiation of tube worms now examined is a relatively recent event. Judging from the cluster of the tube worms, the Slender vestimentifera gen. sp.1 may be included in the genus *Lamellibrachia*.

We have determined the complete amino acid sequences of the four heme-containing chains of *Lamellibrachia* sp.1 440 kDa hemoglobin, very recently [22]. Fig. 4b shows a phylogenetic tree constructed from the four complete sequences (total 576 residues) of *Lamellibrachia* sp.1, *Tylorrhynchus* and *Lumbricus* hemoglobins. This tree must be more reliable than that of Fig. 4a, but the branching pattern for the two trees was quite similar, supporting the accuracy of the tree in Fig. 4a.

Of great interest is the divergence time of tube worms and annelids. The evolutionary rate of hemoglobins is roughly estimated to be constant in vertebrates, and Goodman *et al.* [6] calculated the

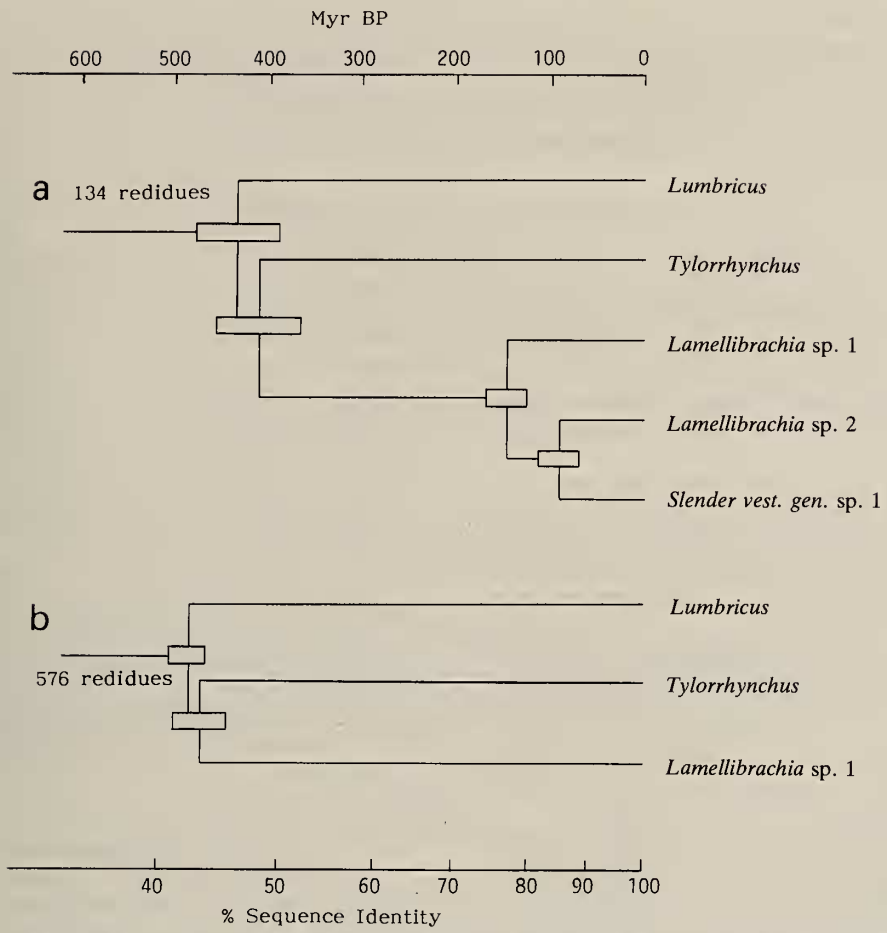

FIG. 4. Phylogenetic trees for the four heme-containing chains of annelid and tube worm hemoglobins. The tree was constructed by an unweighted pair-group clustering method using the Poisson corrected sequence difference matrix. Standard errors at the branching points are indicated as boxes. (a), a tree constructed from the partial amino acid sequences of the four chains compared in Fig. 3 (total 134 residues); (b), a tree from the complete sequences of the four chains consisting of total 576 amino acid residues.

divergence time of human alpha and beta globins with 44% sequence identity to be about 450 million years before present (Myr BP). In addition, the phylogenetic tree constructed from hemoglobin sequences shows a good correlation with that from classical taxonomy [6].

Just as in vertebrates, all of the members belonging to the phylum Annelida expresses abundant hemoglobin, indicating that hemoglobin is a physiologically important molecule. Annelida consists of two major classes, polychaete and oligochaete, but at present it is very difficult to estimate the exact divergence time of the two classes from the poor or incomplete fossil records of annelids. Therefore, to introduce a tentative time scale for the evolution of annelid and tube worm hemoglobins, we used the same evolutionary rate as in vertebrate hemoglobins. Finally, it was estimated that the tube worms, polychaete and oligochaete diverged at almost the same time, about 450 Myr BP, and that the radiation of three tube worms occurred around 100 Myr BP (see Fig. 4). These divergence dates are not unreasonable values, because the fossil records indicate that

most of representatives of the living phyla and classes of invertebrates appeared about 450–500 Myr BP [2]. In addition, Fushitani *et al.* [5] roughly estimated the divergence time of *Lumbricus* and *Tylorrhynchus* to be about 450 Myr BP using hemoglobin sequences.

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